Antioxidant and toxicity screening of extracts obtained from *Cyperus esculentus*

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Abstract: Phytochemicals responsible for toxicity and antioxidant activities of extracts obtained from Cyperus esculentus were investigated. Toxicity test was carried out using Brine shrimp lethality test while in vitro antioxidant activity was detetermined by three methods; scavenging effect on 2,2-diphenyl-1-picryhydrazyl radical (DPPH), inhibition of hydroxyl radical and peroxide oxidation by ferric thiocynate method. Secondary plant metabolites responsible for observed activities in Cyperus esculentus are alkaloids, flavonoids, phenols and glycosides. Brine shrimp lethality test revealed that hexane extract was toxic while the methanol extract was not toxic with a lethality dose (LC_{50}) greater than 1000 μ/ml. The toxicity results support its local use as an antibiotics/antifungal. Both extracts possessed significant antioxidant activity when compared with antioxidant standards; butylated hydroxyl anisole (BHA), ascorbic acid and α-tocopherol used in the assay. The %inhibition was between 98.24% and 95.30% at 0.00625 mg/ml for the n - hexane and methanol extracts respectively. The high antioxidant activity of the plant extracts at low concentration shows that it could be very useful for the treatment of ailments resulting from oxidative stress.

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INTRODUCTION

Cyperus esculentus (Cyperaceae) otherwise known as earth almond, tigernut, yellow or 'Chufa' sedge was first discovered 4000 years ago. It is not actually a nut, but a small tuber and has several health benefits due to the high fibre, proteins and natural sugars content. Several varieties include *Cyperus esculentus* var. *esculentus* (Mediterranean region east of India), *Cyperus esculentus* var. *hermannii* (Florida), *Cyperus esculentus* var. *leptostachyus* (United States), *Cyperus esculentus* var. *macrostachyus* (United States), *Cyperus esculentus* var. *sativus* (Asia). The tubers with a slightly sweet, nutty flavour, are edible compared to the more bitter-tasting tuber of the related purple nutsedge (*Cyperus rotundus*). Tigernuts have excellent nutritional qualities. It is also used as fishing bait and since the tubers contain a reasonable percentage of oil, *C. esculentus* has been suggested as a possible source of biodiesel (Burkill, 1985; Groombridge, 1992; Zohary and Hopf, 2000; Dutta, 2002).

This present study is aimed at investigating the antioxidant activity and toxicity of extracts obtained from *C. esculentus*. Antioxidant chemistry is gaining much attention in recent times due to the involvement of excess free radicals in several pathological situations. Oxidation is induced by reactive oxygen species, radiation or other human activities. Antioxidants with free radical scavenging activities may have great relevance in the prevention and treatment of diseases associated with oxidants (Bors and Saran 1991; Alan and Miller 1996; Potterat, 1997). The antioxidant property will be determined by three methods not yet reported in literature for this plant. Scavenging effect on 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) at 517 nm, hydroxyl radical generated from hydrogen peroxide at 285 nm and peroxide oxidation of extracts from *C. esculentus* at 500 nm using the Ferric thiocyanate method. Butylatedhydroxyanisole (BHA), ascorbic acid and α-tocopherol are used as reference standards (Mensor et al, 2001; Oloyede et al, 2010; Oloyede and Farombi. 2010). Brine shrimp lethality test which is a bench top bioassay for elementary toxicity investigations of bioactive natural products is used for the toxicity studies (Meyer et al., 1980; Keddy et al, 1995). Phytochemical screening was carried out to determine the secondary metabolites present in the plant (Harborne, 1998).

MATERIALS AND METHODS

Chemicals and Reagents:

Hexane, ethyl acetate, methanol, butanol, sodium chloride, copper sulphate pentahydrate, ferric chloride, conc. tetraoxosulphate (VI) acid, conc. HCl,

ammonia solution and chloroform, hydrochloric acid, naphthol, bismuth nitrate, potassium iodide, sodium hydroxide, copper acetate, NaOH, sodium potassium tartarate, potassium chloride, glacial acetic acid, disodium hydrogen phosphate, and dihydrogen potassium phosphate were all BDH general purpose chemicals and distilled prior to use. Dimethylsulphoxide (M&B, England), hydrogen peroxide and 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, butylatedhydroxyanisole (BHA) and α-tocopherol were obtained from Sigma Chemical Co. (St Louis, MO). Brine shrimp larvae eggs were obtained from Ocean Star International, Inc. Company, USA.

Equipment and Apparatus:

Mettler analytical balance H80 (UK), Soxhlet apparatus, Rotavapor RII0 (Buchi, England), Water Bath (Gallenkamp), pH meter (Jenway model), UV-Visible spectrophotometer (Unico1200 & Perkin Elmer lambda 25 models).

Plant collection and identification/Sample preparation

The yellow nutsedge (*Cyperus esculentus*) were obtained at Bodija market, Ibadan, Oyo State, Nigeria in March 2011 and authenticated at the Herbarium of the Department of Botany and Microbiology of University of Ibadan, Nigeria. The seeds were air dried for five weeks and ground into powder using a Thomas-Willey milling machine at the Wood - extraction laboratory of the Department of Chemistry, University of Ibadan, Nigeria. The pulverized sample was stored in a cellophane bag at ambient temperature.

Extraction and Fractionalization of the Plant Sample

N-hexane (4 Lt) and methanol (4 Lt) were separately used to extract 1.4 kg of the pulverized nutsedge using soxhlet apparatus. The extracts obtained were concentrated with the aid of a Bucchi rotavapor and stored in a desiccator prior to further analysis. The samples were screened for the presence of secondary plant metabolites. Thereafter, free radical scavenging activity and toxicity test were carried out on the extracts.

Reference Standards:

Ascorbic acid, butylatedhydroxyanisole (BHA) and α-tocopherol for antioxidant activity. Dimethylsulphoxide (DMSO) for toxicity study.

Phytochemical screening

The extracts obtained above were used to test for the presence of the following plant secondary metabolites; alkaloids, flavonoids, steroids, saponins, phenols, phlobatanins, tannins, glycosides, reducing sugars, anthraquinones, resins and cardiac glycosides (Harborne, 1998).

Antioxidant activities of *Cyperus esculentus* extracts

Scavenging Effect on DPPH

The ability to scavenge the "stable" free radical DPPH or antioxidant activity was determined using the DPPH free – radical scavenging method. A 3.94 mg of 2, 2-diphenyl-1-picryhydrazyl radical (DPPH), a stable radical was dissolved in methanol (100 ml) to give a 100 µm solution. To 3.0 ml of the methanolic solutions of DPPH was added 0.5 ml of each of the extracts with doses ranging from 1.0 mg/ml to 0.0625 mg/ml (Gulcin et al, 2002, Mutee, 2010 and Hatano et al, 1988). The decrease in absorption at 517 nm of DPPH was measured 10 minutes later. The actual decrease in absorption was measured against that of the control and the percentage inhibition was also calculated. The same experiment was carried out on butylated hydroxylanisole (BHA), α-tocopherol and ascorbic acid which are known antioxidants. All test and analysis were run in triplicates and the results obtained were averaged. The radical scavenging activity (RSA) was calculated as the percentage inhibition of DPPH discoloration using the equation below:

$$\%RSA \text{ or } \% \text{ inhibition} = \{(A_{DPPH} - A_S)/A_{DPPH}\} \times 100$$

Where A_S is the absorbance of the sample solution and A_{DPPH} is the absorbance of the DPPH solution.

Scavenging Effect on Hydrogen Peroxide

A solution of 2 mM hydrogen peroxide was prepared in phosphate buffered-saline (PBS) pH 7.4. The extracts at the following concentrations; 0.1 - 0.00625 mg/ml was added to the H_2O_2 solution. Decrease in absorbance of H_2O_2 at 285nm was determined spectrophotometrically 10 minutes later against a blank solution containing the test extract in PBS without H_2O_2. All tests were run in triplicates and averaged (Soares et al., 1997, Oloyede and Farombi, 2010). The same experiment was carried out on Butylatedhydroxyanisole (BHA), ascorbic acid and α-tocopherol which are known antioxidant standards.

Antioxidant activity by ferric thiocynate method

The antioxidant activities of hexane and methanol extracts *C. esculentus* were determined by ferric thiocynate method (Mackie and McCartney, 1989). 10 mg of each extract was dissolved

separately in 99.5% of ethanol and various concentrations (0.00625 - 0.8 µg/ml) were prepared. A mixture of a 2 ml of sample in 99.5% ethanol, 2.0 ml of 2.51% linoleic acid in 99.5% ethanol, 4 ml of 0.05 M phosphate buffer (pH 7.0) and 2 ml of water was placed in a vial with a screw cap and placed in an oven at 60^0C in the dark. To 0.1 ml of this sample solution, 10 ml of 75% ethanol and 0.1 ml of 30% ammonium thiocynate was added. After the addition of 0.1 ml of 2 x 10^{-2} M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance of the red colour which developed was measured in 3 min at 500 nm. The control and standards were subjected to the same procedures as the sample, except that for the control, only solvent was added, and for the standard, sample was replaced with the same amount of Butylatedhydroxyanisole (BHA), ascorbic acid and α-tocopherol (reference compounds) (Oloyede et al., 2010). The inhibition of lipid peroxidation in percentage was calculated using this equation:

% Inhibition = 1 - (A1/A2) X 100

Where A1 was the absorbance of the test sample and A2 was the absorbance of control reaction.

Toxicity analysis

Brine shrimp lethality test

Brine shrimp lethality test (BST) was used to predict the toxicity of the extracts to living organisms. The shrimp's eggs were hatched in sea water for 48 h at room temperature. The nauplii (harvested shrimps) were attracted to one side of the vials with a light source. Solutions of the extracts were made in DMSO, at varying concentrations (1000, 100 and 10 µg/ml) and incubated in triplicate vials with the brine shrimp larvae. Ten brine shrimp larvae were placed in each of the triplicate vials. Control brine shrimp larvae were placed in a mixture of sea water and DMSO only. After 24 h the vials were examined against a lighted background and the average number of larvae that survived in each vial was determined. The concentration at fifty percent mortality of the larvae (LC_{50}) was determined using the Finney computer programme (Meyer et al., 1980; Falope, 1993; Keddy et al, 1995).

RESULTS AND DISCUSSION

Phytochemical screening

Alkaloids, flavonoids, phenol, reducing sugar and glycosides were detected in the methanol extract while alkaloids and reducing sugar were present in the hexane extract while the remaining metabolites were beyond detectable limit. This report agrees with secondary metabolites previously reported in other species of Cyperaceae. These classes of compounds present are known to possess pharmacological activity and therefore could explain the use, traditionally of this plant in ethnomedicine.

Brine Shrimp toxicity test

Brine shrimp toxicity test showed that the n-hexane extract with LC_{50} of 0.0000 µg/ml was toxic while the methanol extract with LC_{50} of 1.7014E + 38 µg/ml was non-toxic (Table 1). Toxicity to Brine shrimp larvae is an indication of medicinal activity in inhibiting cell or tumour growth but their use at high dose should be monitored (Meyer, et al 1982 and Aiyelaagbe, 2010).

Table 1: Brine shrimp lethality test of *C. esculentus* extracts*

Conc.	**1000 µg/ml**		**100 µg/ml**		**10 µg/ml**		**Control**		
Sample	Survivor	Dead	Survivor	Dead	Survivor	Dead	Survivor	Dead	LC_{50} µg/ml
HCE	21	09	24	06	20	10	10	0	0.0000
MCE	30	00	30	00	30	00	10	0	1.7014E+38

*$LC_{50} < 1000$ = Toxic, $LC_{50} > 1000$ = Not Toxic, HCE= n-hexane extract, MCE = methanol extract

Antioxidant Activity

Scavenging effects on DPPH

The reduction in absorbance of DPPH at 517nm caused by the extracts was measured in triplicate after 10min. The percentage inhibition decreases as the concentration decreases. DPPH is known to be a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [20]. *C. esculentus* extracts have moderate activities as free radical scavengers when compared with controls, ascorbic acid, butylatedhydroxylanisole (BHA) and α–tocopherol (Table 2). The two extracts gave %inhibition of 84 - 116% (Fig 1) at 1.0 – 0.0625 mg/ml. The activity is better than that of ascorbic acid and α –tocopherol. N-hexane extract is better in activity than the methanol extract having % inhibition of 116% at 1.0 mg/ml. The high scavenging activity of *C. esculentus* extracts could be linked to the presence of secondary plant product like flavonoids and phenols observed in the phytochemical screening. Flavonoids and phenolics have been shown to be responsible for antioxidant activities of plants (Hatano, et al 1988; Gow-chin and Pin-Der 1994 and Potterat, 1997).

Table 2: Scavenging Effect of *C. esculentus* Extracts on DPPH*

Conc (mg/ml)	HCE	MCE	Ascorbic acid	BHA	α- Tocopherol
1.0	2.0164±0.000	0.1410±0.000	0.0843±0.010	0.0370±0.006	0.6800±0.029
0.5	0.1647±0.000	0.1195±0.000	0.2893±0.128	0.0460±0.008	0.7040±0.003
0.25	0.1272±0.000	0.1174±0.000	0.2977±0.124	0.0483±0.002	0.7047±0.007
0.125	0.1244±0.000	0.1040±0.005	0.3200±0.082	0.0490±0.004	0.7070±0.007
0.0625	0.1172±0.000	0.1187±0.000	0.5147±0.015	0.0650±0.003	0.7207±0.012

*Absorbance measurement of HCE (n-hexane extract), MCE (methanol extract), Ascorbic Acid, BHA and α-Tocopherol at 517nm. Absorbance of DPPH at 517 nm =0.933

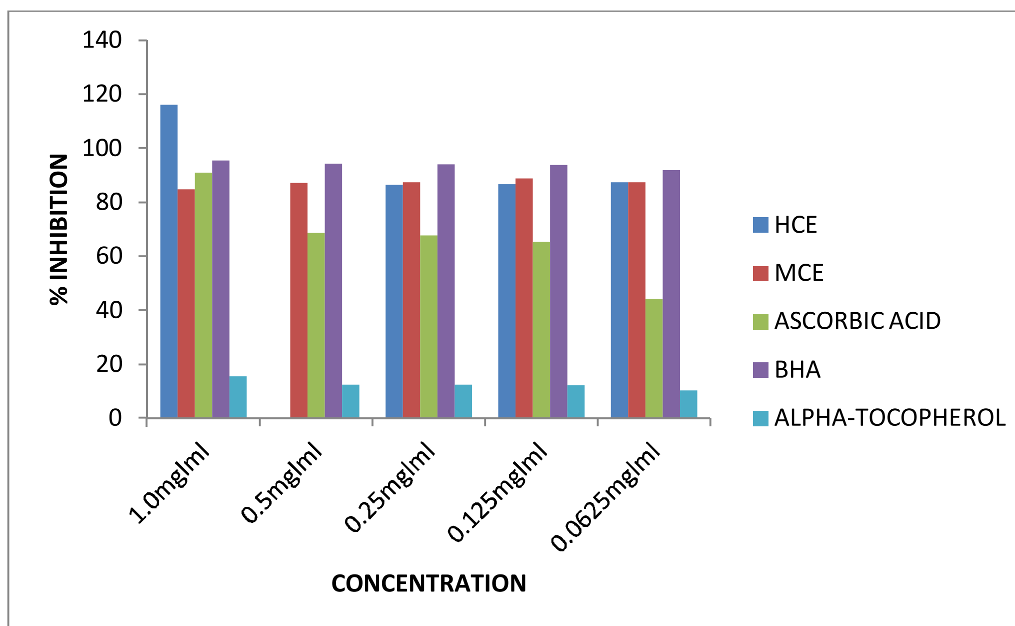

Figure 1: DPPH Free radical scavenging activity of extracts from *C. esculentus*

Scavenging effects on Hydrogen peroxide (H_2O_2)

The scavenging activities of *C. esculentus* extracts and antioxidants standards, ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol on H_2O_2 is shown in Table 3. Scavenging effects on H_2O_2 was measured in triplicates after 10min of incubation at 285nm. At concentration of 1.0 - 0.0065 mg/ml, both extracts had comparable scavenging activities with standards. Methanol extract however scavenged OH radical better than BHA and α-Tocopherol. It has been observed that H_2O_2 is an active - oxygen specie and has potential to produce the highly reactive hydroxyl radical through the Fenton reaction which are usually involved in radical chain reactions (Oloyede and Farombi. 2010).

Table 3: Scavenging Effect of *C. esculentus* Extracts on H_2O_2*

CONC mg/ml	HCE	MCE	ASCORBIC ACID	BHA	ALPHA TOCOPHEROL
0.1	0.076±0.000	0.645±0.013	0.1952±0.001	0.0413±0.016	0.0321±0.045
0.05	0.081±0.000	0.344±0.003	0.2078±0.012	0.0617±0.019	0.0633±0.032
0.025	0.072±0.000	0.209±0.003	1.2645±0.119	0.0740±0.015	0.1552±0.061
0.0125	0.069±0.000	0.181±0.000	2.7586±0.049	0.0947±0.003	0.1807±0.015
0.00625	0.066±0.000	0.177±0.002	2.9236±0.211	0.1126±0.014	0.4940±0.017

*Absorbance measurement of HCE (n-Hexane extract), MCE (Methanol extract), Ascorbic Acid, BHA and α-Tocopherol at 285nm

The %inhibition was between 98.24277% and 95.30227% at 0.00625 mg/ml for hexane and methanol extracts respectively unlike the standards which gave %inhibitions of 94-99% at the highest concentration (0.1 mg/ml) and decreased as the concentration decreases (Fig 2). This result thus revealed that *C. esculentus* has a strong ability as a hydroxyl radical scavenger. The highly polar methanol extract has the highest % inhibition at 0.00625 mg/ml and decreases as the concentration is increased.

Antioxidant activity by Ferric thiocyanate method (FTC)

The FTC method was used to determine the amount of peroxide which oxidized ferrous chloride ($FeCl_2$) to a reddish ferric chloride ($FeCl_3$) pigment. In this method, the concentration of peroxide decreases as the antioxidant activity increases. Hexane, ethyl acetate, butanol and crude methanol extract at various concentration (0.00625 – 0.8 mg/ml), showed antioxidant activities in a concentration dependent manner. However, butanol extracts at all the concentration showed an antioxidant activity (91-96%) better than the activities of all the reference compounds, ascorbic acid, BHA and α- tocopherol (Figure 2). It has been observed that the extract exhibited strong activity with the increase in polarity (with reference to organic solvent), indicating that highly polar organic compounds may play important roles in the activities (Wolf, 2005).

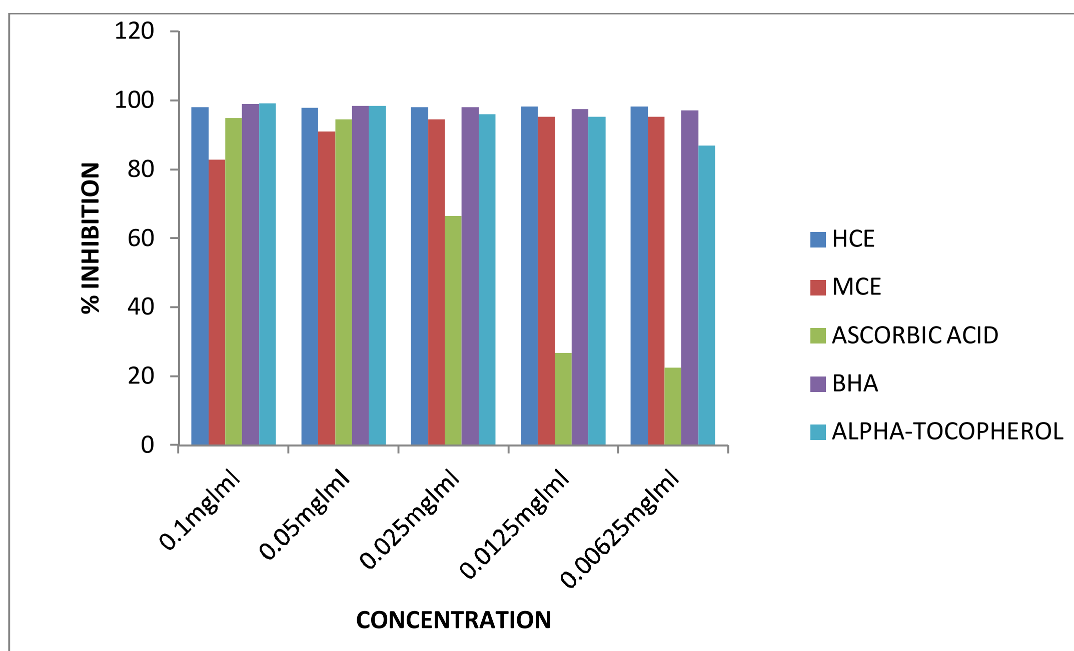

Figure 2: H_2O_2 Free radical scavenging activity of the extracts from *C. esculentus*

Table 4: Peroxide oxidation of extracts from *C. esculentus* at 500 nm using the Ferric thiocyanate method*

CONC mg/ml	HCE	MCE	ASCORBIC ACID	BHA	ALPHA TOCOPHEROL
0.8	0.082±0.007	0.690±0.063	0.173±0.008	0.326±0.006	0.133±0.004
0.4	0.116±0.034	0.628±0.122	0.173±0.008	0.375±0.008	0.164±0.006
0.2	0.153±0.011	0.619±0.004	0.245±0.008	0.431±0.008	0.184±0.009
0.1	0.171±0.009	0.358±0.010	0.275±0.006	0.616±0.005	0.195±0.023
0.05	0.206±0.009	0.348±0.007	0.287±0.050	0.647±0.004	0.294±0.004
0.025	0.355±0.004	0.320±0.007	0.367±0.004	0.653±0.008	0.340±0.069
0.0125	0.370±0.046	0.218±0.005	0.516±0.008	0.747±0.003	0.360±0.005
0.00625	0.606±0.046	0.166±0.011	0.668±0.002	0.750±0.001	0.377±0.008

*Absorbance measurement of HCE (hexane extract) and MCE (Methanol extract), Ascorbic Acid, BHA and α-Tocopherol at 500 nm.

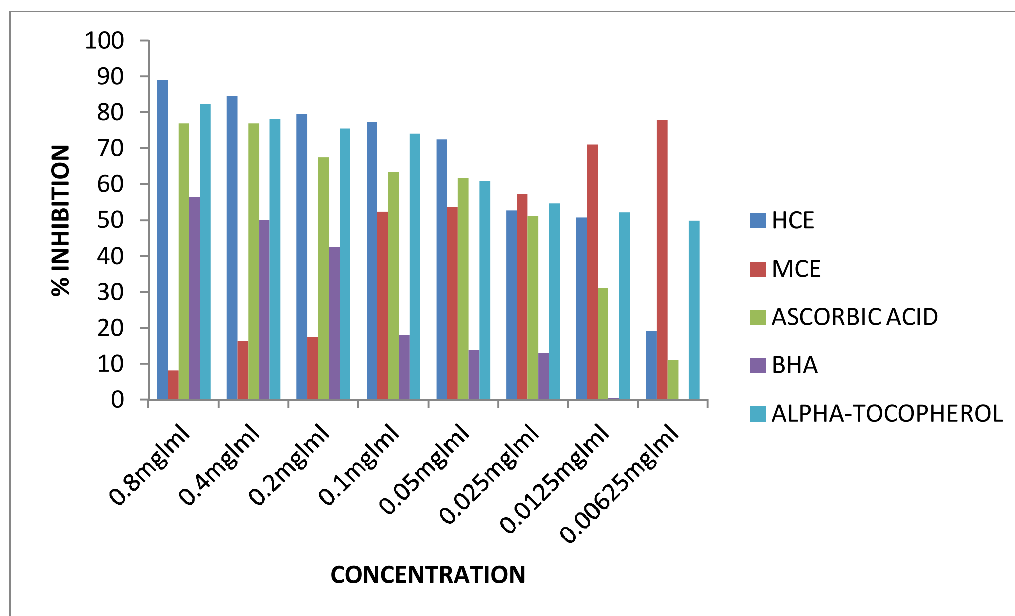

Figure 3: Peroxide oxidation of the extracts from *C. esculentus* and standards at 500 nm measured in triplicate. HCE (hexane extract) and MCE (methanol extract)

CONCLUSION

Phytochemicals found in *C. esculentus* are alkaloids, phenol, flavonoids and glycosides. The brine shrimp toxicity assay showed that the n-hexane extract of the plant was toxic to brine shrimp larvae eggs thus confirming its use as an antimicrobial agent. The plant can also serve as a source of antioxidant agents and can be useful in the therapy of diseases involving cell or tumor growth since it was observed that the plant showed moderate activities in the antioxidant screening. The results of this study underscore the medicinal importance of *C. esculentus* in traditional medicine practice.

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